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Sargassum vulgare Extracts as an Alternative to Chemical Fungicide for the Management of *Fusarium* Dry Rot in Potato

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Abstract

Sargassum vulgare aqueous and methanolic extracts were assessed for their capacity to inhibit *Fusarium sambucinum* and *F. solani*, the most aggressive and frequent causal agents of potato *Fusarium* dry rot in Tunisia. The antifungal potential of these extracts varied according to alga sampling sites, extracts (aqueous or methanolic) and concentrations tested. *F. sambucinum* and *F. solani* mycelial growth inhibition, recorded after 4 days of incubation at 25°C, was estimated at 30.41 and 39.44%, respectively, using aqueous extract of *S. vulgare* at 100 mg/mL. *F. sambucinum* growth was inhibited by more than 32.33% using 100 mg/mL of methanolic extract of alga collected from Tunis. Tested at 50-100 mg/mL, the methanolic extract of alga collected from Tunis had suppressed *F. solani* growth by up to 46%. Methanolic extracts, applied prior tuber inoculation with *F. solani* and *F. sambucinum*, were effective in decreasing disease severity recorded after 21 days of incubation at 25°C. These treatments had lowered the lesion diameter and the rot penetration by 66.65 and 67.51%, respectively, relative to control. The methanolic extract of alga collected from Tunis or Mahdia1 and applied at 50 mg/mL exhibited the highest disease-suppressive ability by decreasing disease severity by 61-63% compared to 3.04-27.13% recorded using carbendazim at the same concentration. Gain of efficiency achieved using *S. vulgare* methanolic extracts (alga from Mahdia1 and Tunis), compared to reference fungicide (applied at 50 mg/mL), was by up to 47% for lesion diameter and more than 62% for fungicide penetration. The chemical analysis of the methanolic extract of *S. vulgare* sampled from site of Mahdia1 using HPLC-DAD revealed the presence of phenolic acids and flavonoids. These results suggest that *S. vulgare* may be explored as potential source of antifungal compounds bioactive against *Fusarium* spp.

Keywords: Brown alga; Disease severity; Dry rot; Carbendazim; *Fusarium sambucinum*; *Fusarium solani*; HPLC-DAD; Methanolic extract

Introduction

Potato (*Solanum tuberosum* L.) is a highly nutritional crop grown worldwide. In 2013, over 376 million tons of potatoes were produced in the world [1] and were stored for long-term to provide a continuous supply of this staple food to consumers and industry. Potato is considered as a promising food to millions of people especially in developing countries.

Pythium aphanidermatum [2], *P. ultimum* [3], *Fusarium* spp. [4-6], *Phytophthora erythroseptica* [5], *Pectobacterium* spp. [7], and *Sclerotium rolfsii* [8], are the most prevalent causal agents of tuber rots in stored potato in Tunisia. *Fusarium* dry rot is caused by a *Fusarium* species complex with *F. sambucinum* and *F. solani* being the most aggressive and devastating ones under Tunisian climate conditions [4,9,10].

Measures for controlling dry rot in storage are limited. There is no commercially grown potato cultivar that is resistant to dry rot in Tunisia [4]. Integrating storage technologies with physical methods and chemical treatments either at harvest or prior tuber storage or before planting could reduce the losses caused by *Fusarium* spp. Dry rot has been managed primarily by reducing tuber bruising and by applying thiabendazole-based fungicides [11]. However, isolates of *F. sambucinum* resistant to thiabendazole and benzimidazoles were detected in Tunisia since 2001 [12]. The occurrence of such resistant strains led to decreased effectiveness of the most widely used chemical treatments for controlling tuber dry rot [13].

Algae produce many antimicrobial (antibacterial and antifungal) products. *Sargassum* genus has been identified as a new and rich source of bioactive compounds. Thus, exploring its antimicrobial efficacy could constitute an environmentally eco-friendly alternative to control plant pathogenic fungi. Algae are used as fertilizers as they can enhance seed germination and seedling growth, increase uptake of plant nutrients, and to provide resistance to abiotic and biotic stresses [14,15]. In the past few decades, many researchers have focused their studies on the exploration of brown algae as a potential source of bioactive materials. *Sargassum* is a brown seaweed abundant in the subtidal regions of Tunisia. This seaweed attracted extensive interest due to its multiple biological activities. *S. vulgare* has been identified as a potential source of wide spectrum of natural substances such as alginate, fucans, sulfated polysaccharide, and polyphenols showing different biological activities [16-18].

Developing alternative control strategies for suppressing potato diseases in storage such as alga-derived extracts as pre-storage tuber treatment is of increasing need. *S. vulgare* was chosen among all seaweeds species to be evaluated for its antifungal potential against the two main causal agents of tuber dry rot, *F. sambucinum* and *F. solani*. In a previous work, we demonstrated the ability of *S. vulgare* extracts to successfully control Pythium leak disease induced by *P. aphanidermatum* and *Fusarium* dry rot incited by *Fusarium oxysporum* f. sp. *tuberosi* [2,6]. The present work was undertaken to assess the effectiveness of these extracts against *Fusarium* spp. based on *in vitro* and *in vivo* bioassays compared to that achieved using a chemical fungicide (Bavistin®, 50% carbendazim).

Materials and Methods

Sargassum vulgare sampling and processing

Alga was sampled during February 2014 from four geographical sites, namely Tunis (N 36°51'53.041"; E 10°21'14.4"), Monastir (N 35°46'47.754"; E 10°47'9.312"), Mahdia1 (N 35°30'15.942"; E 11°4'42.035"), and Mahdia2 (N 35°30'13.278"; E 11°4'34.371") along the Tunisian coast (Mediterranean coast of North Africa). The whole thallus of seaweed samples (Figure 1) were collected by hand picking at a depth of 1 m approximately beneath the sea surface. They were gently rinsed with sea water, transferred into plastic bags and brought to the laboratory.

Figure 1: Morphological features of the macroalga *Sargassum vulgare* (thallus, leaves and vesicles) collected from Tunisian coasts.

S. vulgare samples were thoroughly washed several times with tap water to remove sand particles and marine epiphytes, shade-dried for several days at room temperature, and finally grounded into fine powder using a mixer grinder. Grounded samples were packed and stored at 4°C until use.

Preparation of *Sargassum vulgare* extracts

Algae were subjected to aqueous extraction according to Oryan et al. [19]. Powder samples (of 200 g each) were soaked in 2 L of sterile distilled water (SDW) and maintained under ambient conditions (25 ± 2°C) for 24 h. Extracts were filtered twice through Whatman N°1 sterile filter paper and further sterilized by filtration through micro-filter (0.22 µm pore size).

Collected aqueous extracts were stored at 4°C until future use and used within a week to avoid any chemical alteration.

For methanolic extraction, a 250 g-sample of grounded *S. vulgare* alga was subjected to a series of maceration in methanol (500 mL) for few days under ambient room conditions according to Saidana et al. [20]. After filtration, the solvent was evaporated using a rotary evaporator under reduced pressure (at 60°C). All the methanolic dry residues were quantified and separately dissolved into dimethyl sulfoxide (DMSO) and stored at 4°C until further use.

The yield (%, w/w) from all the dried methanolic extracts was calculated as follows:

Yield (%)=$(w_1/w_2) \times 100$ where w_1 is the weight of the extract after evaporation of solvent (methanol), and w_2 is the weight of alga powder. Methanol extractions yielded over 12%.

Plant material

Healthy and undamaged potato (*S. tuberosum* L.) tubers cv. Spunta, the mostly grown cultivar in Tunisia, were used for tuber inoculation and treatments' testing. They were previously stored in darkness at 6°C for one month before use and brought to room ambient conditions few hours before use.

Prior to inoculation, tubers were thoroughly washed to remove adhering soil and superficially disinfected with a 10% (v/v) sodium hypochlorite solution during 5 min then rinsed with SDW and air dried until use.

Fusarium spp. culture and inoculum preparation

Fusarium spp. (*F. sambucinum* and *F. solani*) isolates used in the present study were gratefully provided by the Phytopathology laboratory of the Regional Center of Research on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia. Isolates were originally recovered from potato tubers exhibiting *Fusarium* dry rot symptoms.

Pathogen cultures were initiated from stock cultures maintained at 4°C. *Fusarium* isolates were cultured on Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulphate (300 mg/L). Their virulence was regularly preserved by bimonthly inoculation of freshly wounded tubers and re-isolation on PDA plates.

Spore suspensions were prepared by culturing isolates in Potato Dextrose Broth (PDB) at 25°C and under continuous shaking at 150 rpm. After 7 days of incubation, the obtained liquid cultures were filtered through two layers of cheesecloth to remove mycelium and then through two layers of Whatman No. 1 sterile filter paper. The final conidial concentration in the filtrates was adjusted to 10^7 conidia /mL using a Malassez cytometer. An equal volume of each conidial suspension (100 µL) from each *Fusarium* species was used for preparation of the mixed inoculum for tuber inoculation.

Test of the response of target *Fusarium* species to carbendazim and *S. vulgare* extracts

Carbendazim (Bavistin®; 50% carbendazim) and *S. vulgare* aqueous and methanolic extracts were screened *in vitro* for their comparative activity against *F. sambucinum* and *F. solani* using the poisoned food technique on PDA medium amended with streptomycin sulphate (300

mg/L). Appropriate amounts (1-100 mg/mL) of each tested extract (aqueous and methanolic) or carbendazim were added to molten PDA medium. Chemical fungicide- and extract-amended PDA media were aseptically poured into a Petri plates (9 cm in diameter). SDW and DMSO were used as negative controls. After medium solidification, three agar plugs (6 mm in diameter), cut from 7-day-old PDA cultures of target pathogen (*F. sambucinum* and *F. solani*), were equidistantly placed in each Petri plate.

The mean diameter of *F. sambucinum* and *F. solani* colonies was noted after 4 days of incubation in the dark at 25°C. The percentage of mycelial growth inhibition was calculated using the following formula: I=(C-T/C) × 100 where I: pathogen growth inhibition (in %), C: diameter of pathogen colony in control plates and T: diameter of pathogen colony in treated plates. Three replicates (plates) were used for each individual treatment. The whole experiment was repeated twice.

Test of the disease-suppression ability of *S. vulgare* methanolic extracts

A mixed inoculum composed of *F. sambucinum* and *F. solani*, being the most aggressive species complex responsible for serious tuber decays during storage [4], was used for *in vivo* trials. Disinfected potato tubers were wounded at two sites along the tuber longitudinal axis using a sterile cork borer. The induced wound, of 6 mm in diameter and depth, serves as an infection site.

Tuber treatments were performed 4 h (preventive treatment) before pathogen challenge by injecting 100 µL (at different concentrations 1-100 mg/mL) of each *S. vulgare* methanolic extract, collected from the four sampling sites, in the occasioned wounds.

Tuber inoculation was made by deposing 100 µL of a mixed conidial suspension (*F. sambucinum* and *F. solani*). Positive control tubers were inoculated with mixed inoculum and treated with a similar volume of SDW whereas the negative control ones were uninoculated and treated with SDW.

Two inoculation sites were used per tuber and each individual treatment was replicated five times. The whole experiment was repeated twice. After inoculation, tubers were placed in plastic bags to maintain humidity and incubated for 21 days at 25°C.

At the end of the experiment, *Fusarium* dry rot severity was estimated through the external and the internal extent of the induced rot. External symptomatic lesions were measured through two perpendicular diameters of the lesion and the mean diameter was calculated for each inoculation site using the following formula:

Mean lesion diameter (mm)=(d1+d2)/2 where d1 and d2 two perpendicular diameters. Dry rot severity was also estimated internally through the extent of dry rot within tuber tissues. Tubers were cut along the longitudinal axis across the inoculation sites and the maximal width (w) and depth (d) of the rotted tissue were measured. Rot penetration within tubers was calculated based on Lapwood et al. [21] formula as follow:

Rot penetration (mm)=(w/2+(d-6)/2).

Comparative efficacy test of the most bioactive methanolic extracts and carbendazim in controlling disease

Carbendazim-based fungicide was applied 4 h before pathogen challenge by injecting, in the occasioned wounds, 100 µL of the fungicide (prepared at two concentrations 50 and 100 mg/mL). The selected *S. vulgare* methanolic extracts (the most active ones and concentrations), based on their ability to suppress *Fusarium* dry rot disease, were also re-evaluated for their disease-suppressive potential as compared to carbendazim on potato tubers co-inoculated with *F. sambucinum* and *F. solani*.

Tuber treatments at the selected concentrations and inoculation were performed as described above. Positive control tubers were inoculated with mixed inoculum and treated with SDW. The negative control ones were non-inoculated and similarly treated using SDW only. Two inoculation sites were used per tuber and each individual treatment was replicated five times. The whole experiment was repeated twice. After incubation at 25°C for 21 days, mean lesion diameter and penetration were calculated as described above.

Analysis of polyphenols using HPLC-DAD system

The most active *S. vulgare* methanolic extract, exhibiting higher efficacy than carbendazim in suppressing *Fusarium* dry rot, was selected for the identification of its content in phenolic compounds using a high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD system). The DAD detector was set to a scanning range of 340 nm. Column temperature was maintained at 25°C. For two extracts (mobile phase of chromatography: ethanol and water), the injected sample volume was 2 µL and the flow-rate of mobile phase was 0.4 mL/min.

Identification of phenolic compounds was performed by comparing their retention times with those of the standard phenolic compounds (pure phenol standards including 20 phenolic acids and 20 flavonoids) which were dissolved in methanol at a concentration of 1 mg/mL and injected under the same chromatographic conditions. Evaluation of each standard was repeated three times.

Statistical analyses

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software for Windows version 20.0. A factorial analysis of variance (ANOVA) was performed to determine the significance of the main factors and their interactions using a completely randomized factorial design with two factors. Mean separations were performed according to Student-Newman-Keuls's and /or LSD tests (at $P \leq 0.05$).

Results

Response of target *Fusarium* species to carbendazim-based fungicide

Analysis of variance performed for data of both target *Fusarium* species indicated that their mean colony diameters, recorded after 4 days of incubation at 25°C, varied significantly (at $P \leq 0.01$) upon tested carbendazim concentrations. Response of both *Fusarium* species to carbendazim-based treatments varied depending on concentrations tested (Figure 2) as indicated by the significant interaction noted between the two fixed factors. In fact, *F. sambucinum* was found to be resistant to the tested chemical fungicide even at the highest concentration tested (100 mg/mL) where pathogen colony was around 1 cm in diameter. As compared to the untreated control, mean colony diameter was lowered by 78.08% using carbendazim at 100 mg/mL compared to 37.42% achieved with concentration of 1 mg/mL. However, *F. solani in vitro* growth was totally suppressed by

carbendazim-based treatments applied at 5-100 mg/mL and pathogen colony growth did not exceed 1 cm in diameter at the lowest concentration tested. Thus, *F. solani* was found to be sensitive to the tested chemical fungicide as the mean colony diameter of pathogen was decreased by 83.82%, versus the untreated control, when grown on PDA amended with 1 mg/mL of carbendazim (Figures 2 and 3).

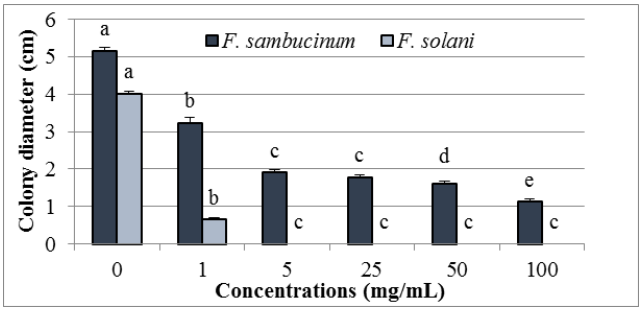

Figure 2: Response of *Fusarium sambucinum* and *F. solani* to different concentrations of carbendazim noted after 4 days of incubation at 25°C as compared to the untreated controls. Carbendazim is the active ingredient of Bavistin® (50% carbendazim, chemical fungicide). Concentration 0 mg/mL: (Control) PDA medium unamended with carbendazim. For each *Fusarium* species, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels' test (at $P \leq 0.05$). LSD (*Fusarium* species × Carbendazim concentration tested)=0.12 cm at P=0.05.

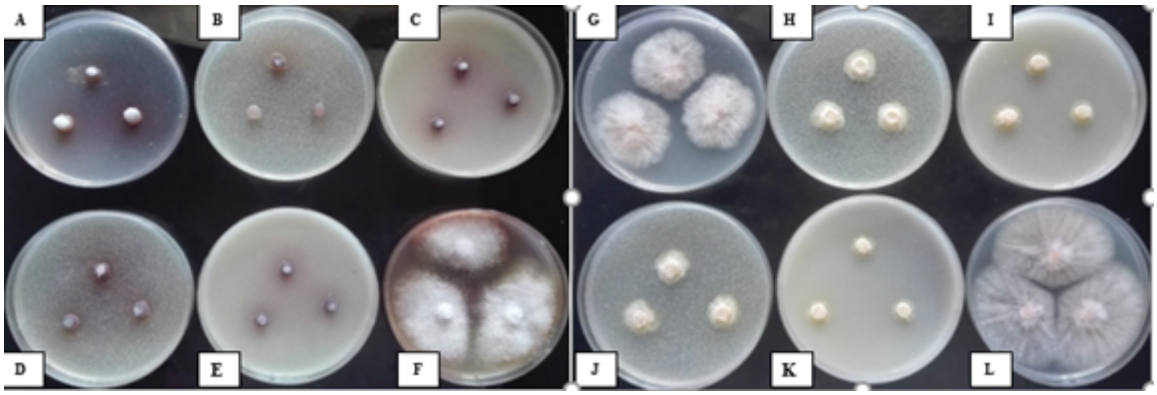

Figure 3: *Fusarium solani* (left) and *F. sambucinum* (right) colonies grown on PDA supplemented with different concentrations (1-100 mg/mL) of carbendazim (Bavistin®, 50% carbendazim, reference fungicide) recorded after 4 days of incubation at 25°C. (A) and (G): Carbendazim tested at 1 mg/mL; (B) and (H): Carbendazim tested at 5 mg/mL; (C) and (I): Carbendazim tested at 25 mg/mL; (D) and (J): Carbendazim tested at 50 mg/mL; (E) and (K): Carbendazim tested at 100 mg/mL; (F): *F. solani* control grown on PDA medium amended with sterile distilled water (SDW);(L): *F. sambucinum* control grown on PDA medium amended with SDW.

Response of target *Fusarium* species to *Sargassum vulgare* aqueous extracts

ANOVA analysis, performed separately for data of each *Fusarium* species, showed a highly significant (at $P \leq 0.01$) variation in average colony diameters of both pathogens, noted after 4 days of incubation at 25°C, depending on alga sampling sites, concentrations tested, and their interactions. In fact, as shown in Figure 4A, all aqueous extracts, whatever their sampling sites and tested concentrations, had significantly suppressed *F. sambucinum* radial growth, by 6.90-30.41% as compared to the untreated control. Tested at 50-100 mg/mL, *S. vulgare* aqueous extract sampled from site of Tunis had suppressed *F. sambucinum* growth by 24.76-26.65% respectively, relative to control, compared to 11.91-13.17% noted using concentrations ranging between 1 and 25 mg/mL. Similarly, aqueous extract of *S. vulgare* removed from site of Monastir was also found to be more effective when used at 100 and 50 mg/mL leading to 18.50 and 21.00% decrease in pathogen growth compared to 12.85% recorded at 1 mg/mL. Growth inhibition induced by alga aqueous extract sampled from Mahdia1 was estimated at 30.41% when tested at 100 mg/mL compared to 23.20-7.21% recorded with the other concentrations. For alga collected from Mahdia2 site, the highest inhibitions, by about 25.71-22.26% over control, were noted using extracts at 100 and 50 mg/mL compared to 14.73-7.21% achieved with the other concentrations.

It should be indicated that for all concentrations tested combined, aqueous extracts of alga removed from Tunis and Mahdia1 were found to be the most effective in inhibiting *F. sambucinum* radial growth. For

data of all sampling sites combined, the highest decreases in pathogen mycelial growth, by about 25.31% versus control, was recorded with the highest concentration (100 mg/mL).

Data given in Figure 4B showed that, excepting aqueous extracts of *S. vulgare* collected from Mahdia1 and applied at 1 and 5 mg/mL and those from Mahdia2 used at 1 mg/mL, all the remaining aqueous extracts tested, whatever their sampling sites and concentrations, had significantly decreased *F. solani* radial growth by 5.18- 39.44% versus control.

S. vulgare aqueous extracts collected from Tunis tested at 50 and 100 mg/mL had inhibited *F. solani* growth by 39.44 and 36.65%, respectively, compared to 8.76-25.90% noted at 1-25 mg/mL. Aqueous extract of alga removed from Monastir was found to be more active against *F. solani* when applied at 100 mg/mL (growth inhibited by 39.44%) than at concentrations ranging between 1 and 50 mg/mL (16.33-28.29%). It is also the case of extracts of *S. vulgare* removed from Mahdia1 and Mahdia2 where the highest growth inhibition (32.67 and 31.47%, respectively) was noted at 100 mg/mL compared to 5.18-6.77% recorded at 1 mg/mL.

Overall, for all concentrations combined, aqueous extracts of alga removed from Tunis and Monastir were found to be the most effective in suppressing *F. solani* radial growth. Additionally, for all the sampling sites combined, the highest inhibition of pathogen growth was recorded at 100 mg/mL.

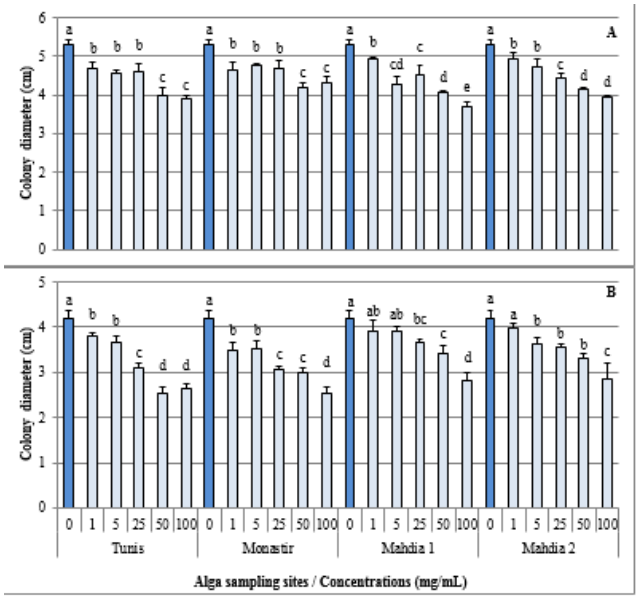

Figure 4: Variation of the antifungal activity of *Sargassum vulgare* aqueous extracts against *Fusarium sambucinum* (A) and *F. solani* (B) depending on alga sampling sites and tested concentrations noted after 4 days of incubation at 25°C. Concentration 0 mg/mL: (Control) PDA medium unamended with aqueous algal extracts. For each sampling site, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels' test (at $P \leq 0.05$). LSD (Sampling sites × Concentrations tested)=0.14 cm (A) or 0.15 cm (B) at P=0.05.

Response of target *Fusarium* species to *Sargassum vulgare* methanolic extracts

ANOVA analysis revealed a highly significant (at $P \leq 0.01$) effect of the treatments tested on *Fusarium* spp. radial growth, noted after 4 days of incubation at 25°C, according to alga sampling sites and tested concentrations. A significant interaction was also noted between these last two factors. All tested methanolic extracts, whatever alga sampling sites, and concentrations tested had significantly reduced *F. sambucinum* (Figure 5A) and *F. solani* (Figure 5B) *in vitro* growth, by 8.16-32.33 and 8.24-49.06%, respectively, compared to the untreated control.

As shown in Figure 5A, the methanolic extract of *S. vulgare* removed from Tunis was found to be more active when applied at 100 mg/mL where *F. sambucinum* mycelial growth was inhibited by 32.33%, relative to control, compared to 9.67-21.75% recorded with the other concentrations. For alga removed from Monastir, the methanolic extract applied at 100 mg/mL had inhibited pathogen radial growth by about 24.47% over control compared to 8.76-13.60% noted at concentrations ranging between 1 and 50 mg/mL. Methanolic extract of S. vulgare sampled from Mahdia1 displayed its highest antifungal potential against *F. sambucinum*, expressed by 26.89% decrease in pathogen growth, when applied at 100 mg/mL compared to 8.76% noted at 1 mg/mL. Similarly, *F. sambucinum* growth was lowered by 28.40% using the methanolic extract of alga collected from Mahdia and applied at 100 mg/mL and by 8.16 to 18.73% using the other concentrations.

It can be concluded from data given in Figure 5A that for all sampling sites combined, the tested methanolic extracts were more active when used at 100 mg/mL. Also, for all concentrations combined, *S. vulgare* methanolic extract sampled from Tunis was the most effective in reducing *F. sambucinum* growth (Figure 6).

Data presented in Figure 5B revealed that the methanolic extract of alga sampled from Tunis was more active when applied at 100 mg/mL, where pathogen growth was lowered by 37.83%, relative to the control, compared to 18.35% noted at 1 mg/mL. Those of alga sampled from Monastir and applied at 50-100 mg/mL had inhibited *F. solani* radial growth by more than 36% and by 11.24-20.97% when used at 1-25 mg/mL. Methanolic extract of *S. vulgare* removed from Mahdia1 was also more effective at 50-100 mg/mL leading to 49.06 and 46.07% decrease in pathogen radial growth compared to 8.24-34.08% achieved with concentrations ranging between 1 and 25 mg/mL. *F. solani* mycelial growth was decreased by 27.72-31.46% using the extract of alga removed from Mahdia2 at 50-100 mg/mL compared to 10.86-20.97% recorded at the concentrations ranging between 1 and 25 mg/mL.

It can be deduced from data given in Figure 5B that for all concentrations combined, the methanolic extract of alga removed from Mahdia1 was the most active toward *F. solani* (Figure 7). In addition, and for all sampling sites combined, *S. vulgare* methanolic extracts were most active when applied at 50-100 mg/mL where pathogen growth was inhibited by 35.86-38.11% versus the untreated control.

Methanolic extracts of *S. vulgare* collected from the different sampling sites along Tunisian costs were selected as being the most bioactive ones against *F. sambucinum* and *F. solani* where the growth inhibition rates were estimated at 8.16-32.33 and 8.24-49.06% (Figures 5A and 5B) compared to 6.90-30.41 and 5.18-39.44% (Figures 4A and 4B), respectively, obtained using aqueous extracts. These selected

methanolic extracts were further screened for their ability to suppress *Fusarium* dry rot development and severity.

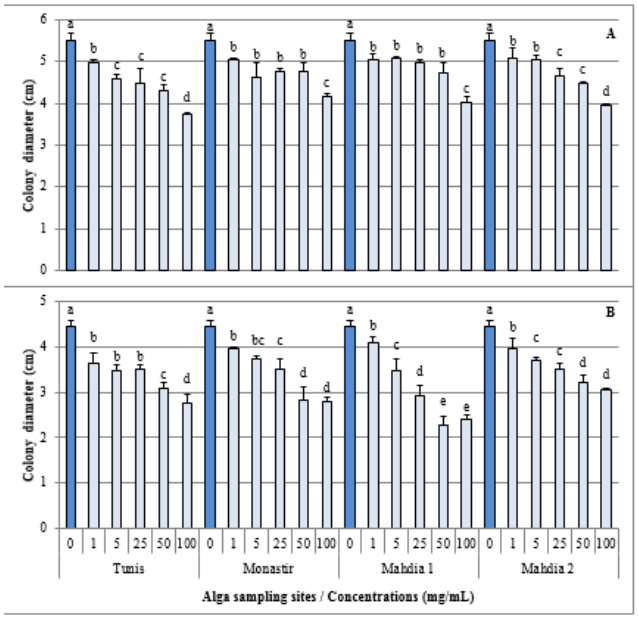

Figure 5: Variation of the antifungal activity of *Sargassum vulgare* methanolic extracts against *Fusarium sambucinum* (A) and *F. solani* (B) depending on alga sampling sites and tested concentrations noted after 4 days of incubation at 25°C.Concentration 0 mg/mL: (Control) PDA medium unamended with methanolic algal extracts. For each sampling site, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels' test (at $P \leq 0.05$). LSD (Sampling sites × Concentrations tested) = 0.16 cm (A) or 0.15 cm (B) at P = 0.05. Concentration 0 mg/mL: (Control) PDA medium unamended with methanolic algal extracts. For each sampling site, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels' test (at $P \leq 0.05$). LSD (Sampling sites × Concentrations tested)=0.16 cm (A) or 0.15 cm (B) at P=0.05.

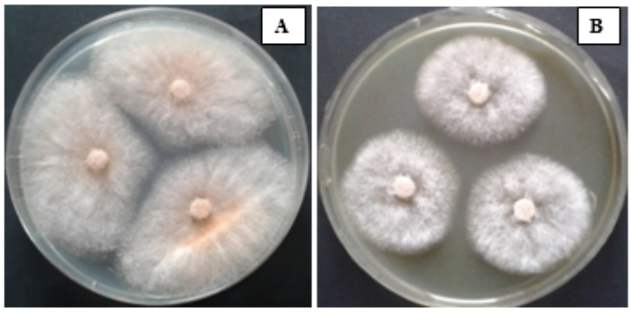

Figure 6: *Fusarium sambucinum* colonies grown on PDA amended with 100 mg/mL of methanolic extract of *Sargassum vulgare* removed from Tunis (B) compared to the untreated control (A) recorded after 4 days of incubation at 25°C.

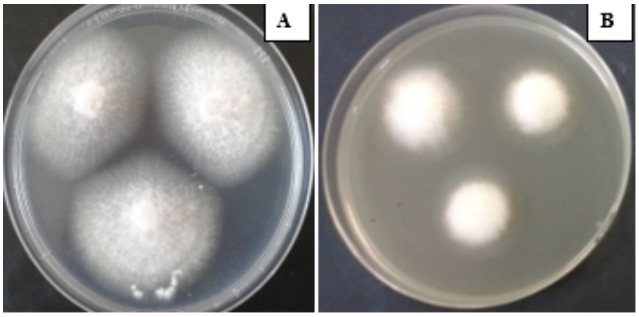

Figure 7: *Fusarium solani* colonies grown on PDA amended with 50 mg/mL methanolic extracts of *Sargassum vulgare* removed from Mahdia1 (B) compared to the untreated control (A) recorded after 4 days of incubation at 25°C.

Disease-suppressive ability of *Sargassum vulgare* methanolic extracts

ANOVA analysis performed for both *Fusarium* dry rot severity parameters (lesion diameter and rot penetration) showed that these two parameters, noted after 21 days of incubation at 25°C, varied significantly (at $P \leq 0.05$) depending on alga sampling sites (Tunis, Monastir, Mahdia1 and Mahdia2), tested concentrations (1-100 mg/mL) and their interactions.

Data given in Figure 8 showed that, whatever alga sampling sites and tested concentrations, all tested *S. vulgare* methanolic extracts were effective in reducing disease severity compared to Fusarium spp.-inoculated and untreated control. In fact, these treatments had significantly reduced by 35.22-66.65% the lesion diameter of dry rot induced by a mixed infection with *F. sambucinum* and *F. solani*.

As shown in Figure 8, treatment of potato tubers using the methanolic extract of *S. vulgare* collected from Tunis was effective in decreasing the lesion diameter of *Fusarium* dry rot by 63.60-66.65% when applied at 25-100 mg/mL compared to 35.22-55.42% achieved with the other concentrations. Lesion diameter was also lowered by 61.89-63.15% following tuber treatment with 50 to 100 mg/mL of methanolic extract of alga sampled from Monastir site compared to 42.64-52.15% obtained at 1-25 mg/mL. For alga collected from Mahdia1, the tested methanolic extract was found to be more effective in reducing dry rot severity by 59.77-61.39% when used at concentrations ranging between 5 and 100 mg/mL as compared to 51.74% noted at 1 mg/mL. Those of *S. vulgare* sampled from Mahdia2 had lowered the rot lesion diameter by 51.57-57.00% when applied at 5-100 mg/mL while 43.84% was recorded following treatments with 1 mg/mL.

It should be highlighted that for concentrations combined (Figure 9), methanolic extracts of alga collected from sites Tunis and Mahdia1 were found to be the most effective in reducing lesion diameter as compared to the two others.

Moreover, for all sampling sites combined, *S. vulgare* methanolic extracts were found to be more active at 50-100 mg/mL where the lesion diameter was reduced by 61.17-62.87% compared to 43.36% noted at 1 mg/mL.

Data illustrated in Figure 10 showed that, whatever alga geographical origin and concentrations tested, all screened methanolic extracts were effective in significantly suppressing rot penetration by 25.05 to 67.51% versus the inoculated and untreated control.

As shown in Figure 10, tuber treatment of potato s with the methanolic extract, of *S. vulgare* sampled from Tunis, used at 50 and 100 mg/mL led to 67.11-63.60% decrease in dry rot penetration and 58% using the other concentrations. Those from alga removed from Monastir had suppressed dry rot penetration by 32.94-46.58% when tested at concentrations varying from 5 to 100 mg/mL compared to 25.05% obtained at 1 mg/mL. Interestingly, treatments with methanolic extract of *S. vulgare* collected from Mahdia1 induced a strong inhibition of rot penetration, estimated at 55.90-66.99%, when used at concentrations ranging between 5 to 100 mg/mL compared to 42.16% recorded at 1 mg/mL. Concerning extracts from alga removed from Mahdia2, their ability to suppress dry rot severity was estimated at 56.75-65.69% when applied at 25-100 mg/mL and at 30.20% when used at 1 mg/mL.

Overall, as compared based on alga sampling sites whatever the concentrations tested, methanolic extracts of *S. vulgare* removed from sites of Tunis and Mahdia1 showed the highest disease suppression ability as compared to the two other sites (Figure 11). Moreover, for all sampling sites combined, the tested methanolic *S. vulgare* extracts were more efficient in suppressing disease when used at concentrations of 50 and 100 mg/mL where rot penetration was decreased by 58.70-61.80% compared to 38.88% recorded at 1 mg/mL.

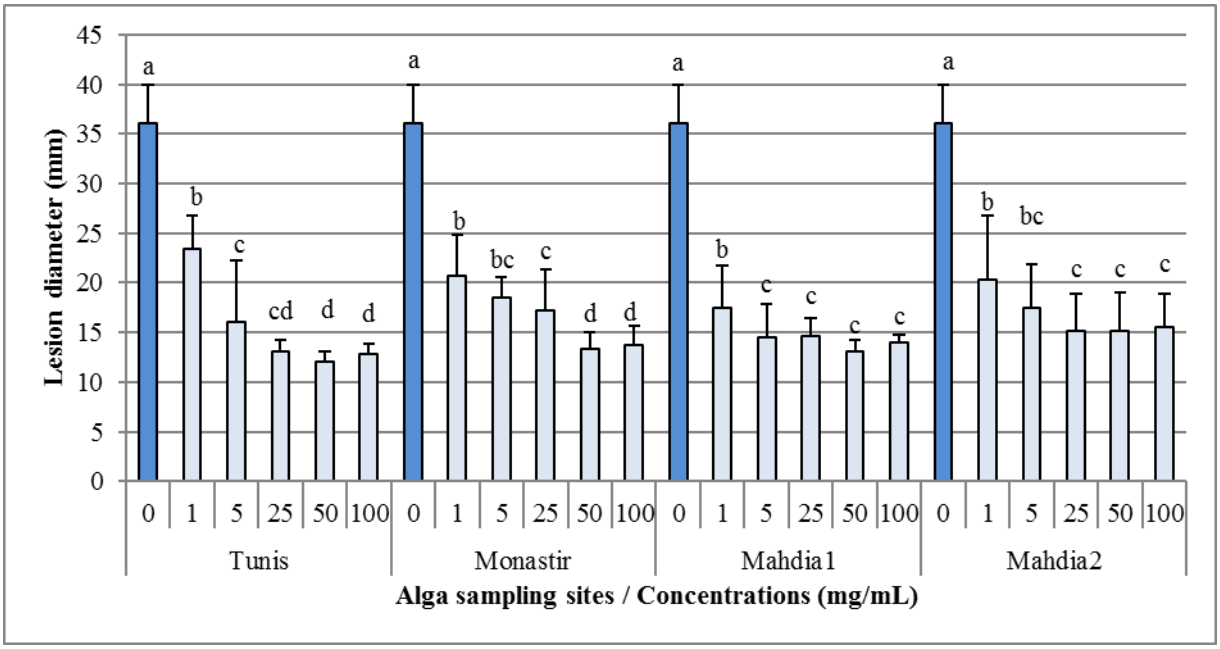

Figure 8: Disease-suppressive ability of *Sargassum vulgare* methanolic extracts, as measured by lesion diameter, depending on alga sampling sites and tested concentrations noted after 21 days of incubation at 25°C. For each sampling site, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels test (at $P \leq 0.05$). LSD (Alga sampling sites × Concentration tested)=2.66 mm at P=0.05.

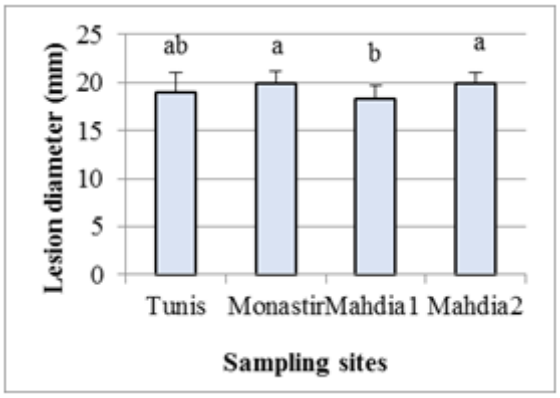

Figure 9: Variation of *Fusarium* dry rot severity (lesion diameter) noted after 21 days of incubation at 25°C on *Fusarium* spp.-inoculated and treated potato tubers depending on *Sargassum vulgare* sampling sites (data for all concentrations combined). Bars sharing the same letter are not significantly different according to Student-Newman-Keels test (at $P \leq 0.05$).

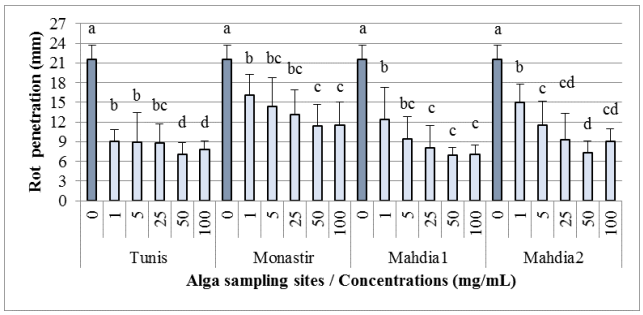

Figure 10: Disease-suppressive ability of *Sargassum vulgare* methanolic extracts, as measured by rot penetration, depending on alga sampling sites and tested concentrations, noted after 21 days of incubation at 25°C. For each sampling site, concentrations sharing the same letter are not significantly different according to Student-Newman-Keels test (at $P \leq 0.05$). LSD (Alga sampling sites × Concentration tested)=2.75 mm at P=0.05.

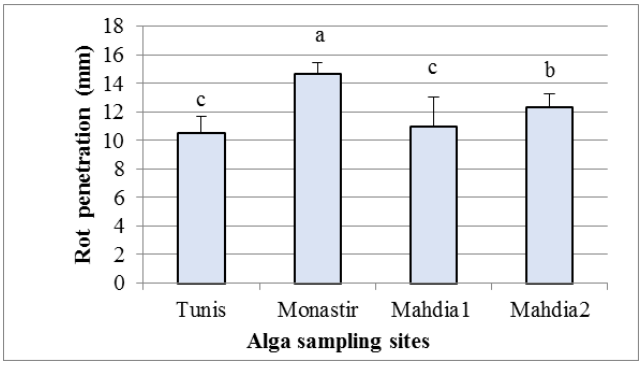

Figure 11: Variation of *Fusarium* dry rot severity (rot penetration) noted after 21 days of incubation at 25°C on *Fusarium* spp.-inoculated and treated potato tubers depending on *Sargassum vulgare* sampling sites (data for all concentrations combined). Bars sharing the same letter are not significantly different according to Student-Newman-Keels test (at $P \leq 0.05$).

Comparative efficacy of the most effective *S. vulgare* methanolic extracts and carbendazim in controlling *Fusarium* dry rot

Methanolic extracts of *S. vulgare* sampled from sites of Tunis and Mahdia1 and applied at 50 mg/mL were selected for comparison of their efficacy with that achieved using a chemical reference fungicide i.e., carbendazim (Bavistin®, tested at two concentrations 50 and 100 mg/mL).

ANOVA analysis performed for disease severity parameters (lesion diameter and rot penetration) showed a significant (at $P \leq 0.01$) variation depending on tested treatments (two *S. vulgare* methanolic extracts, two fungicide treatments and the untreated control). Treatment with methanolic *S. vulgare* extracts sampled from sites of Mahdia1 and Tunis and applied at 50 mg/mL were more effective in reducing lesion diameter, by 61.69 and 64.72% compared to 27.13-47.84% recorded with carbendazim applied at 50-100 mg/mL, respectively (Figure 12A). As estimated based on dry rot penetration, potato tubers treated with methanolic extract of *S. vulgare* removed from Mahdia1 and Tunis sites exhibited important disease suppressive abilities by decreasing rot penetration by 63.90 and 67.15%, compared to control, when tested at 50 mg/mL compared to 3.04 and 16.61% noted using carbendazim at 50 and 100 mg/mL, respectively (Figure 12B).

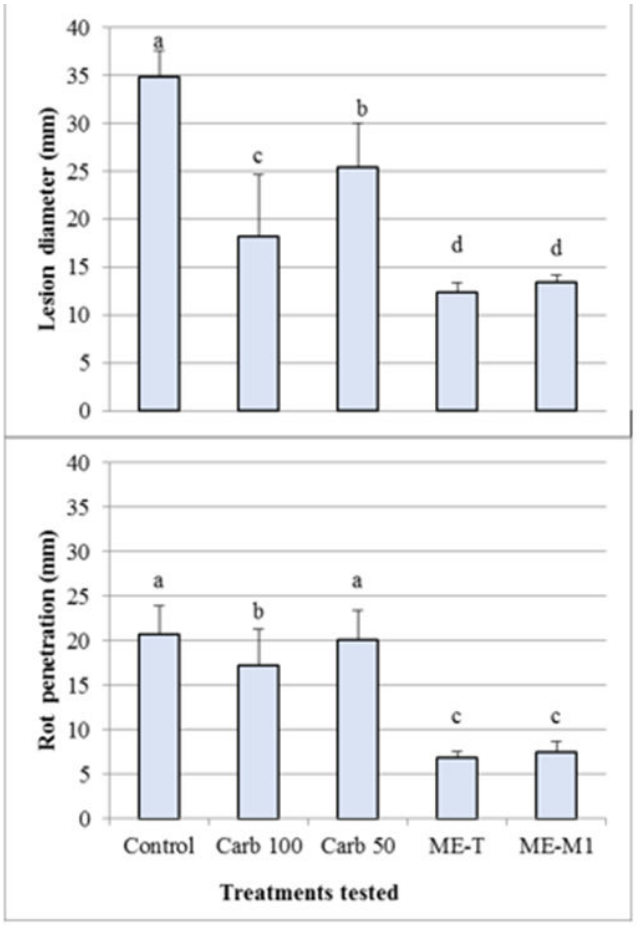

Figure 12: Comparative efficacy of the most effective *Sargassum vulgare* methanolic extracts and carbendazim in controlling *Fusarium* dry rot noted after 21 days of incubation at 25°C. Carb100: Potato tubers treated with 100 mg/mL of carbendazim; Carb50: Potato tubers treated with 50 mg/mL of carbendazim; ME-T: Potato tubers treated with 50 mg/mL of methanolic extract of *S. vulgare* sampled from Tunis; ME-M1: Potato tubers treated with 50 mg/mL of methanolic extract of *S. vulgare* sampled from Mahdia1. Tuber inoculation was performed using a mixed inoculum composed of *Fusarium sambucinum* and *F. solani*. Treatment sharing the same letter are not significantly different according to Student-Newman-Keels' Multiple Range test (at $P \leq 0.01$).

Treatment with *S. vulgare* methanolic extracts removed from sites of Mahdia1 and Tunis and applied at the concentration of 50 mg/mL allowed a gain of efficiency, when compared to carbendazim applied at 100 mg/mL, by about 26.55 and 32.36% for lesion diameter and by 56.71 and 60.60% for rot penetration, respectively. A higher efficiency of these methanolic extracts was noted, when compared to carbendazim-based treatment tested at 50 mg/mL, and was expressed by 47.43 and 51.58% decrease in the lesion diameter and by about 62.77 and 66.12% in the rot penetration (Figures 12 and 13).

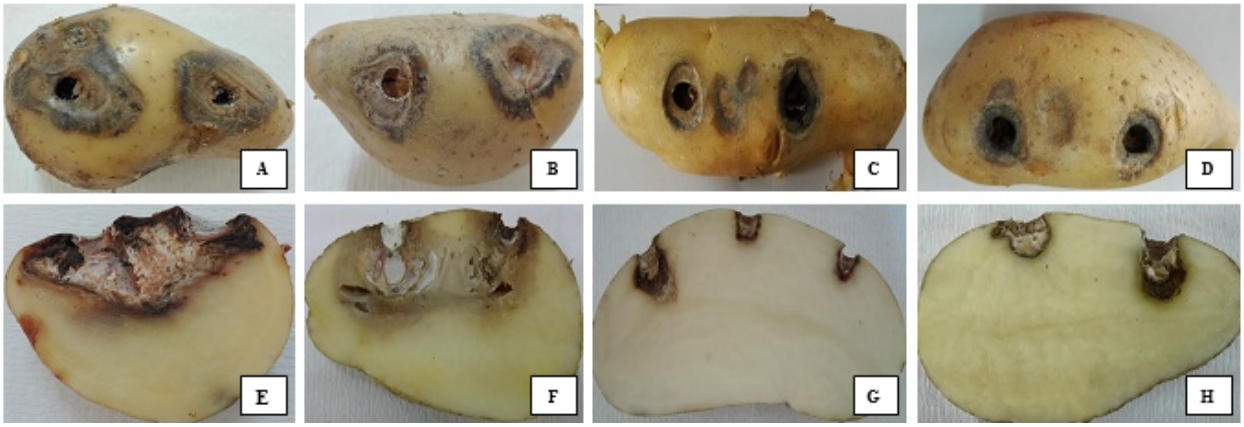

Figure 13: Comparative efficacy of the most bioactive methanolic extracts and carbendazim in controlling *Fusarium* dry rot severity recorded after 21 days of incubation at 25°C compared to pathogen-inoculated and untreated control. (A, E): *Fusarium* spp.-inoculated and untreated control; (B, F): Tubers inoculated with *Fusarium* spp. and treated with carbendazim at 50 mg/mL (Bavistin®, 50% carbendazim); (C, G): Tubers inoculated with *Fusarium* spp. and treated with *S. vulgare* (removed from *Tunis*) methanolic extract used at 50 mg/mL; (D, H): Tubers inoculated with *Fusarium* spp. and treated with *S. vulgare* (removed from Mahdia1) methanolic extract used at 50 mg/mL.

Chemical analysis of polyphenols in the most effective *S. vulgare* methanolic extract using HPLC-DAD

S. vulgare methanolic extract sampled from the site Mahdia1, being the most active in suppressing *Fusarium* dry rot disease more efficiently than the reference fungicide i.e., carbendazim, was subjected to chemical analysis. In fact, analysis of polyphenols performed using HPLC-DAD chemical profiling revealed the presence of 30 compounds. The identification of these compounds was achieved by comparing the retention times of standard phenolic compounds (40 compounds) with those of the injected *S. vulgare* methanol extract.

Identification and quantification of the polyphenolic compounds present in the methanol extract collected from Mahdia1 site, obtained under optimal conditions, are shown in Table 1. Thirteen compounds were separated at different retention times viz., 2.35,2.54, 2.70, 2.86, 3.01, 3.13, 3.70, 5.35, 5.54, 6.02, 6.23, 6.45, 6.91, 7.49, 7.76, 8.44, 8.88, 9.75, 14.46, 14.77, 16.96, 21.88, 24.93, 27.21, 27.99, 28.44, 29.07, 29.58, 30.04, and 30.54 min, respectively.

Peak n°	Retention time (min)	Area	Compound	Percentage (%)
8	5.35	400.71	Ascorbic acid	14.04
16	8.44	11.01	Gallic acid	0.39
18	9.75	14.45	Resorcinol	0.51
19	14.47	24.75	Catechin-hydrate	0.87
20	14.77	12.89	Epigallocatechin	0.45
21	16.96	18.65	Epicatechin-3-O-gallate	0.65
22	21.88	17.15	Oleuropein	0.6
23	24.93	90.13	Luteolin	3.16
24	27.21	16.6	Cirsimaritin	0.58

Table 1: Main phenolic acid and flavonoid compounds identified in the methanolic extract of the brown alga *Sargassum vulgare* sampled from Mahdia1 and analyzed by HPLC-DAD.

HPLC-DAD analysis revealed the presence of the following compounds: ascorbic acid (14.04%), gallic acid (0.39%), resorcinol (0.51%), catechine hydrate (0.87%), epigallocatechine (0.45%), epicatechine-3-O-gallate (0.65%), oleuropein (0.60%), luteolin (3.16%), and cirsimaritin (0.58%). The other peaks (n° 1-7, 9-15, 17, and 25-30) were not identified. The major chemical constituents detected in the methanol extract obtained from Mahdia1 were ascorbic acid and three other compounds were not identified (at 2.86, 6.23, 29.07 min). The performed HPLC-DAD analysis allowed identification of 21.25% of compounds only.

Discussion

There is an increased need for tuber protection using effective and environmentally safe alternatives to minimize postharvest losses. In view of this continuing research for new nature-derived alternatives to chemical control of fungal plant pathogens, the present study was performed to evaluate the potential of *S. vulgare extracts* collected from four Tunisian geographical sites to inhibit the *in vitro* and *in vivo* growth of *F. sambucinum* and *F. solani* as compared to a chemical fungicide used as reference i.e., carbendazim.

The present investigation demonstrated that *S. vulgare* aqueous extracts had affected, with different levels, the mycelial growth of the target pathogens. The highest inhibition rate of *F. sambucinum* growth, by more than 30.41%, was noted with aqueous extract sampled from site of Mahdia1 applied at concentration of 100 mg/mL. In the same way, the mycelial growth of *F. solani* was lowered by up to 39.44% when grown on PDA medium amended with aqueous extract of alga sampled from Tunis and used at 100 mg/mL. These results are in accordance with our previous findings where *Pythium aphanidermatum* and *F. oxysporum* f. sp. *tuberosi* mycelial growth

were also decreased by more than 28%, compared to control, using *S. vulgare* aqueous extract at 50 mg/mL [2,6]. In fact, several researches have shown the antifungal activity of aqueous extracts from *Sargassum* species against numerous pathogenic fungi. For instance, aqueous extract of *S. tenerrimum* (at 16%) was effective in reducing the mycelial growth of *Rhizoctonia solani* and *F. oxysporum* by more than 74% and that of *Macrophomina phaseolina* by 86% [22]. In addition, *S. bindarri* and *S. swightii* aqueous extracts have inhibited *F. oxysporum* mycelial growth by 70% [22]. However, *S. glaucescens*, *S. swartzii*, *S. wightii*, and *S. tennirrimum* aqueous extracts were found to be inactive against *F. solani* [23-25]. Also, Chbani et al. [26] demonstrated that aqueous extract of *S. vulgare* tesed at 200 mg/mL was totally inactive against *Penicillium digitatum*).

Data from the current investigation revealed that *S. vulgare* methanolic extracts possessed the highest inhibitory activity toward *F. sambucinum* and *F. solani* depending on alga sampling sites and tested concentrations. In fact, at 100 mg/mL, the methanolic extract S. vulgare removed from Tunis had inhibited *F. sambucinum* growth by 32.33% compared to 9.67% recorded using the same extract at 1 mg/mL. In addition, an important inhibition of *F. solani* mycelial growth by 36.07-49.06% was induced by the methanolic extract of alga removed from Tunis tested at 50-100 mg/mL. In line with our results, *S. vulgare* methanolic and chloroformic extracts tested at 50 mg/mL had totally inhibited mycelial growth of *F. monilliforme* [27]. In addition, cyclohexane extract of *S. vulgare* applied at 50 mg/mL had strongly inhibited *F. oxysporum* mycelial growth. However, *S. vulgare* ethanolic, ethyl acetate and chloroformic extracts were slightly active against this pathogen whereas, acetonic one was totally inactive [28]. Also, methanolic and dichloromethanolic extracts of *S. vulgare* were found to be inactive against *F. solani* and *P. digitatum*, respectively [26,29]. In addition, Ambreen, et al. [30] demontrated that *S. ilicifolium*, *S. lanceolatum* and *S. swartzii* ethanolic extracts applied at 2 mg/mL were totally inactive *against F. oxysporum*, *F. solani*, *M. phaseolina* and *R. solani*.

The highest inhibition of in vitro mycelial growth of both target pathogens was achieved using alga methanolic extracts. Selected based on their ability to suppress mycelial growth of *Fusarium* spp., these extracts were further evaluated for their potential to suppressive dry rot incited by a mixed infection by *F. sambucinum* and *F. solani*. Interestingly, under extremely favorable conditions for Fusarium spp. development, and when used at concentration of 50 mg/mL, the *S. vulgare* methanolic extracts removed from Tunis and Mahdia1 sites and applied preventively showed important disease-suppressive ability by reducing by up to 66 and 67% the rot lesion diameter and the rot penetration relative to the pathogen-inoculated and untreated controls, respectively. The dry rot-suppressive ability displayed by the two selected *S. vulgare* methanolic extracts was confirmed and was found to be higher that achieved using the reference chemical treatment (Bavistin®, 50% carbendazim). In fact, tested at 50 mg/mL, methanolic extracts from *S. vulgare* samples collected from Tunis and Mahdia1 sites were found to be effective in reducing not only lesion diameter (by up to 61% compared to 27.13% noted with carbendazim at the same concentration) but also the rot penetration (by up to 63% compared to only 3.4% recorded with the chemical fungicide). Results from the current study obviously demonstrated that preventive application of these extracts led to reduced *Fusarium* rot severity more efficiently than carbendazim. Several reports have also indicated the effectiveness of algal extracts in suppressing soilborne plant pathogens. For instance, Ammar et al. [6] showed that when applied (at 100 mg/mL) prior to *F. oxysporum* f. sp. *tuberosi* challenge, treatment with chloroformic and methanolic extracts exhibited the highest disease-suppressive effects versus the inoculated and untreated control. Methanolic extract of *S. vulgare* applied at concentration of 1 mg/mL was effectively sufficient for getting protection of potato tubers against Pythium leak disease caused by *P. aphanidermatum*, where rot penetration was lowered by more than 82% [2]. In this sense, leaf sprays of tomato plants with *S. fusiforme* extract had strongly decreased *Botrytis cinerea*, *Phytophthora infestans* and *Oidium* spp. infections and disease severities [31]. However, Chbani et al. [26] found that *S. vulgare* aqueous and dichloromethanolic extracts failed to suppress citrus fruits infection caused by *P. digitatum* even when applied at 200 mg/mL. This indicates that the spectrum of activity of these extracts is species-specific (alga and/or target pathogens) and concentration-dependent.

Moreover, Moorthy et al. [32] affirmed that the antifungal activity depends on method of extraction of secondary metabolites, their solubility, temperature, polarity of solvents, crude extract concentration and especially in pathogen-specific. Several authors demonstrated that methanolic extract exhibited higher antimicrobial activity than the other organic extracts [2,6,33,34]. Methanol was selected as being the most appropriate solvent for extraction of antimicrobial compounds for red, green and brown seaweeds [23,35].

From the current findings, *S. vulgare* extracts display an antifungal potential site-dependent. In fact, variation in the antifungal activity depending on distant sampling sites (as is the case of Tunis, Monastir and Mahdia sites in the current study) as well as close areas (Mahdia1 and Mahdia 2). This may be assigned to exposure of these algae to biotic and abiotic stresses such as UV, temperature, salinity, light, hydro-dynamism or nutrients, growth and maturity of thallus which are known to influence chemical defense production in brown seaweeds that in turn lead to the formation of phyto-components with variable antifungal activities [36,37].

Methanolic extracts were found to possess not only antifungal potential *in vitro* against target pathogens but also protect potato tubers from *Fusarium* spp. infection. For instance, methanolic extract collected from Tunis and Mahdia1 had reduced mycelial growth of *F. sambucinum* (by 21.75 and 14.20%) and that of *F. solani* (by 30.34 and 49.06%), respectively, when applied at 50 mg/mL. However, Sbaihat et al. [31] demonstrated the absence antifungal potential of *S. fusiforme* extract against *B. cinerea*, *Phytophthora infestans* and *Oidium* spp. infections in tomato but resistance reactions were induced in tomato plants because of *S. fusiforme* extract applications.

Utilization of algal *Sargassum* extracts can be an efficient tool to manage the most aggressive *Fusarium* species (*F. sambucinum* and *F. solani*) under Tunisian climate conditions. Gain of efficiency allowed by *S. vulgare* methanolic extracts removed from Mahdia1 and Tunis when compared to carbendazim applied at 50 mg/mL exceeded 47% for lesion diameter and up to 62% for rot penetration.

Phenolic compounds are one of the most widely occurring groups of phytochemicals known by their important biological activities in alga. Many studies have demonstrated the richness of *S. vulgare*, *S. filipendula*, *S. dublicatum*, *S. crassifolium*, and *S. binderi* in phenolic compounds [2,38]. In a recent study, Ammar et al. [2] also showed, using HPLC-DAD chemical profiling, the presence of phenolic acids and flavonoid compounds in the methanolic extracts of *S. vulgare* sampled from Tunis. Chemical classes, including phenols, terpenes, acetogenins and fatty acids were also detected in *S. vulgare* ethanolic extract [39,40].

Conclusion

This study showed the *in vitro* and *in vivo* inhibitory effects of *S. vulgare* extracts collected from Tunisian coastal locations, against *F. sambucinum* and *F. solani* causing tuber dry rot. The importance of this work is high as it is the first report on ability of seaweed extracts to suppress severity of this economically important potato disease and probably other potato pathogens more efficiently that carbendazim used as reference chemical treatment.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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